Itani, N., Kuramitsu, S., Ikeda, K., & Hamaguchi, K. (1975) *J. Biochem. (Tokyo) 78*, 705-711.
Jolles, P., & Jolles, J. (1984) *Mol. Cell. Biochem. 63*, 165-189.
Kuramitsu, S., Ikeda, K., Hamaguchi, K., Fujio, H., Asano, T., Miwa, S., & Nishida, T. (1974) *J. Biochem. (Tokyo) 76*, 671-683.
Lehrer, S. S., & Fasman, G. D. (1967) *J. Biol. Chem. 242*, 4644-4651.
Matsumura, M., Becktel, W. J., & Matthews, B. W. (1988) *Nature 344*, 406-410.
Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., & Horiuchi, T. (1987) *Protein Eng. 1*, 327-332.
Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., & Matsubara, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1-5.
Nitta, K., & Sugai, S. (1989) *Eur. J. Biochem. 182*, 111-118.
Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211-2217.
Ogasahara, K., & Hamaguchi, K. (1967) *J. Biochem. (Tokyo) 61*, 199-210.
Pace, L. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1-43.
Parson, S. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1623-1629.
Roxby, R., & Tanford, C. (1971) *Biochemistry 10*, 3348-3352.
Rupley, J. A. (1964) *Biochim. Biophys. Acta 83*, 245-255.
Rupley, J. A., Butler, L., Gerring, M., Hartdegen, F. J., & Pecoraro, R. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1088-1095.
Spassov, V. Z., Karshikov, A. D., & Atanasov, B. P. (1989) *Biochim. Biophys. Acta 999*, 1-6.
Tanford, C. (1962) *J. Am. Chem. Soc. 84*, 4240-4247.
Tanford, C., Pain, R., & Otchin, N. (1966) *J. Mol. Biol. 15*, 489-504.
Taussig, R., & Carlson, M. (1983) *Nucleic Acids Res. 11*, 1943-1954.
Toh-e, A., Ueda, Y., Kakimoto, S., & Ohshima, Y. (1973) *J. Bacteriol. 113*, 727-738.
Yamada, H., & Imoto, T. (1981) *Carbohydr. Res. 92*, 160-162.
Yamada, H., Ito, Y., Fukumura, T., & Imoto, T. (1985a) *Anal. Biochem. 146*, 71-74.
Yamada, H., Ueda, T., Kuroki, R., Fukumura, T., Yasukochi, T., Hirabayashi, T., Fujita, K., & Imoto, T. (1985b) *Biochemistry 24*, 7953-7959.
Yutani, K., Ogasahara, K., Tsujita, T., & Sugino, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4441-4444.

A Hybrid Ribulosebisphosphate Carboxylase/Oxygenase Enzyme Exhibiting a Substantial Increase in Substrate Specificity Factor†

Betsy A. Read and F. Robert Tabita*
Department of Microbiology and The Biotechnology Center, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210-1292

Received January 24, 1992; Revised Manuscript Received March 31, 1992

ABSTRACT: Two hybrid ribulose-1,5 bisphosphate carboxylase/oxygenase (RubisCO) enzymes were constructed using RubisCO small subunit genes (*rbcS*) from two eucaryotic marine organisms, *Cylindrotheca* sp. N1 and *Olisthodiscus luteus*, cloned downstream of the RubisCO large subunit gene (*rbcL*) of the cyanobacterium *Synechococcus* PCC 6301. The expression products synthesized by *Escherichia coli* JM107 (pVTAC223 and pANOLI) were purified and examined by polyacrylamide gel electrophoresis and compared to the purified products generated by *E. coli* MV1190 (pBGL710), containing cyanobacterial *rbcL* and *rbcS* genes. Both *Cylindrotheca* and *Olisthodiscus* small subunits were able to assemble in vivo with the *Synechococcus* large subunit octamer to form heterologous hexadecameric L_8S_8 enzymes, the pVTAC223 and pANOLI hybrid enzymes, respectively. Like the *Synechococcus* RubisCO, the hybrid enzymes were rapidly activated by Mg^{2+} plus HCO_3^-, even in the presence of RuBP. The hybrid enzymes, however, were considerably more sensitive to the competitive inhibitor 6-phosphogluconate. Detailed kinetic analysis indicated that while the carboxylase activity of both chimeric enzymes was severely reduced, in the case of the pVTAC223 hybrid enzyme, the degree of partitioning between carboxylation and oxygenation was increased nearly 60% relative to the *Synechococcus* RubisCO. Other kinetic properties, including the Michaelis constants for the gaseous substrates and RuBP, were altered in the hybrid proteins. These studies also led to the finding that the substrate specificity factor of the *Cylindrotheca* RubisCO is unusually high.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)[1] is a bifunctional enzyme that is fundamental to the metabolism of all plants, algae, and most photosynthetic bacteria. The enzyme assumes a pivotal role in catalyzing the initial reaction in two competing metabolic pathways: (i) photosynthetic carbon fixation and (ii) photorespiration. Because oxygen inhibits carboxylation and photorespiration leads to the oxidation of reduced carbon with no apparent selective advantage to the plant, it is thought that photosynthetic efficiency and net productivity might be enhanced by

† This work was supported by a grant from the National Institutes of Health (GM-24497).

* Correspondence should be addressed to this author at the Department of Microbiology, The Ohio State University.

[1] Abbreviations: RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; *rbcS*, small subunit gene; *rbcL*, large subunit gene; IPTG, isopropyl β-D-thiogalactopyranoside; TEM, 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; TEMMB, 25 mM TEM, pH 8.0, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/92/0431-5553$03.00/0

genetically or chemically altering RubisCO to preferentially increase its potential to catalyze carboxylation over oxygenation. RubisCO has thus been the subject of intensive investigation, with the eventual goal of gaining an understanding of how the structure of the enzyme correlates with the carboxylase and oxygenase activities.

RubisCO is found predominantly as a hexadecameric protein composed of eight large subunits and eight small subunits, with four small subunits attached to both the top and bottom of a large subunit octameric core (Andersson et al., 1989; Knight et al., 1990). The large subunit has a molecular mass of about 52 kDa and in eucaryotes is chloroplast-encoded, while the small subunit is nuclear-encoded in plants and green algae and typically has a molecular mass of 13 kDa (Andrews & Lorimer, 1987; Tabita, 1988). A number of catalytic functions have been assigned to the large subunit, whose amino acid sequence is highly conserved across species of higher plants, green algae, and cyanobacteria (≥75% identity) (Hudson et al., 1990). The RubisCO large subunit of "nongreen" algae and chemolithoautotrophic and phototrophic bacteria appear to be highly homologous (Hwang & Tabita, 1991; Gibson et al., 1991); however, there is considerably less homology to the closely related higher plant, green algal, and cyanobacterial large subunits. Sites for activation and catalysis are confined to the large subunit, which is also thought to determine the degree of partitioning between carboxylation and oxygenation (Andrews & Lorimer, 1985). The large subunit is subject to posttranslational modification and, in addition, binds well-known effector molecules such as 6-phosphogluconate and ribulose 1,5-bisphosphate (RuBP). Unlike the large subunit, the function of the small subunit is largely unknown. It is obviously required for maximal catalytic activity (Andrews, 1988; Gutteridge, 1991; Lee & Tabita, 1990; Smrcka et al., 1991), and recently small subunits were shown to influence various kinetic properties of the L_8 octameric core (Lee et al., 1991; Read & Tabita, 1992; Smrcka et al., 1991), properties which are consistent with their potential to influence the catalytic site of the enzyme (Knight et al., 1990; Schneider et al., 1990). The direct involvement of small subunits in the catalytic mechanism has not been demonstrated.

Delineating the function of the small subunit is complicated by a second form of RubisCO, isolated from certain purple nonsulfur bacteria, that is comprised exclusively of large subunits (Tabita, 1988; Tabita & McFadden, 1974; Gibson & Tabita, 1977). *Rhodobacter sphaeroides* possesses a form II RubisCO that is an oligomer of four to six large subunits (Gibson & Tabita, 1977), while *Rhodospirillum rubrum* possesses the simplest structural form of RubisCO, a large subunit homodimer (Tabita & McFadden, 1974). Although the k_{cat} values of these single-subunit RubisCOs are comparable to that of the predominant two-subunit form, the substrate specificity factor (a measure of the enzyme's ability to descriminate between CO_2 and O_2) is substantially lower than that of L_8S_8 RubisCO molecules (Jordan & Ogren, 1981a).

To test the involvement of the small subunit in RubisCO catalysis, several hybrid enzymes were constructed (Andrews et al., 1984; Andrews & Lorimer, 1985; Lee et al., 1991). Andrews et al. (1984) combined isolated subunits from the cyanobacterium *Synechococcus* ACMM 323 and the prochlorophyte *Prochloron*, while Andrews and Lorimer (1985) combined *Synechococcus* large subunits with small subunits isolated from the *Spinacea oleracea* (spinach) holoenzyme in vitro. Lee et al. (1991) constructed heterologous enzymes by cloning bacterial small subunit genes (*rbcS*) from *R. sphaeroides* and *Alcaligenes eutrophus* behind the large subunit gene (*rbcL*) from *Synechococcus* sp. PCC 6301 (*Anacystis nidulans*). In each instance, the heterologous enzymes were found to be assembled into a catalytically active, presumably hexadecameric RubisCO. However, when the specificity factor was measured, the partitioning between carboxylation and oxygenation was identical for hybrids and their respective parent enzymes (Andrews & Lorimer, 1985; Lee et al., 1991), suggesting that large subunits alone govern the specificity factor.

In the present investigation, the properties of two other hybrid RubisCO enzymes are reported. Chimeric proteins were expressed in *Escherichia coli* using eucaryotic *rbcS* from a marine diatom, *Cylindrotheca* sp. strain N1, and a marine brown alga, *Olisthodiscus luteus*; in each case, the algal *rbcS* was inserted downstream from the *Synechococcus* 6301 *rbcL* gene. Various biochemical characteristics and kinetic parameters of the hybrid enzymes were compared with those of the enzyme containing homologous large and small subunits. Most notable was the substitution of *Cylindrotheca* small subunits, which conferred a 60% increase in the CO_2/O_2 substrate specificity factor relative to the *Synechococcus* enzyme. These results demonstrate that, under certain circumstances, the RubisCO small subunit may play an important role in the partitioning between carboxylase and oxygenase functions. These studies have also led to the finding that the substrate specificity factor of RubisCO isolated from *Cylindrotheca* sp. strain N1 is unusally high.

Experimental Procedures

Materials. RuBP was prepared according to the previously described method (Horecker et al., 1958). [1-^{3}H]RuBP was synthesized from [2-^{3}H]glucose using hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphoribulokinase (Jordan & Ogren, 1981b); phosphoglycolate phosphatase was purified from tobacco leaves (Jordan & Ogren, 1981b). $NaH^{14}CO_3$ and [2-^{3}H]glucose were purchased from Amersham Corp (Arlington Heights, IL). All enzymes and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Bacterial Strains and Plasmids. Escherichia coli strains JM107 (Maniatis et al., 1982) and MV1190 (Kunkel et al., 1987) were used as host strains for the heterologous enzyme studies. Plasmid pBGL710 (Lee & Tabita, 1990) contains the 2.2-kb *Pst*I fragment of plasmid pCS75 (Tabita & Small, 1985) encoding the *rbcL* and *rbcS* genes of *Synechococcus* sp. PCC 6301 in plasmid pTZ18R (Mead et al., 1986). Plasmid pVTAC223 (Hwang & Tabita, 1989) contains the *Synechococcus rbcL* gene on a 1.5-kb *Bam*HI fragment and the *rbcS* gene of *Cylindrotheca* sp. N1 on a 2.1-kb *Bam*HI/*Eco*RI fragment. Plasmid pANOLI was constructed to include a 1.5-kb *Pst*I fragment, containing the *rbcL* gene of *Synechococcus*, upstream of the 0.8-kb fragment encoding the *rbcS* gene from *Olisthodiscus luteus*, derived from plasmid pOCPEO.8 (Newman et al., 1989).

Cell Culture and Preparation of Crude Extracts. Cultures of *E. coli* MV1190 (pBGL710) encoding the *rbcLrbcS* genes of *Synechococcus* 6301, *E. coli* JM107 (pVTAC223), or *E. coli* JM107 (pANOLI), containing the heterologous *rbc* genes from *Cylindrotheca* sp. N1 and *Olisthodiscus luteus*, respectively, were grown in 1-L batch cultures using Luria–Bertani (Maniatis et al., 1982) medium containing 100 μg/mL ampicillin. Cells were grown at 37 °C with vigorous shaking. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM during exponential growth to induce the *lac* promoter, driving expression of the recombinant

RubisCO. Cultures were allowed to grow for an additional 12–14 h prior to harvesting cells.

Harvested cells were washed in TEM buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β-mercaptoethanol), resuspended in TEM containing 1 mM phenylmethanesulfonyl fluoride (PMSF), and then lysed by passage through a French pressure cell at 1010 atm (1 atm = 1.013 × 10^5 Pa). Cell debris was removed by centrifugation, first at 12000*g* for 10 min and then at 100000*g* for 1 h to yield a crude extract.

Cylindrotheca sp. strain N1 was cultured in Asp-2 medium supplemented with 500 mg/L $Na_2SiO_3 \cdot 5H_2O$, 2 g/L $NaNO_3$, 16 μg/L vitamin B_{12}, and 1.5 mg/L vitamin B_6 (Estep et al., 1978). Batch cultures of 1.5 L were illuminated by a bank of two 15-W deluxe Cool White fluorescent lamps and gassed with 1% CO_2 in air at room temperature. RubisCO was isolated from cells during late exponential growth, when the culture reached a density of 8 g wet weight/L. Harvested cells were resuspended in 250 mM Bicine–NaOH (pH 7.6) buffer containing 10 mM DTT, 1 mM PMSF, and 5 mM EDTA. A crude extract was then obtained as described above.

Purification of RubisCO Proteins. The recombinant *Synechococcus* RubisCO was purified according to previously described methods (Lee & Tabita, 1990). Crude extracts were loaded onto Green-A–agarose columns equilibrated with TEMMB (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$). The enzyme was eluted using a 0–1 M NaCl gradient in TEMMB. Active fractions were pooled and precipitated using ammonium sulfate to 70% saturation. The precipitate was resuspended and dialyzed against TEM buffer prior to being loaded onto a 0.2–0.8 M sucrose step gradient (Tabita & Small, 1985). Fractions of 1.5 mL were collected and assayed for RubisCO activity. The hybrid enzymes, containing the large subunits from *Synechococcus* and small subunits from either *Cylindrotheca* sp. strain N1 or *Olisthodiscus luteus*, were purified in the same manner. The purity of the preparations and the integrity of the enzymes were monitored using both nondenaturing and sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE).

Cylindrotheca N1 RubisCO was purified according to the method of Hwang and Tabita (1989). The crude extract was fractionated with ammonium sulfate, and the precipitate obtained from the fractionation of 25–50% saturation was resuspended and dialyzed in 50 mM Bicine–NaOH, pH 7.6, containing 1 mM DTT and 1 mM EDTA. The enzyme was further purified by Green-A column chromatography and sucrose step gradient centrifugation as previously described. The integrity and purity of the preparation were again determined by nondenaturing and SDS–PAGE. The extinction coefficient of the purified protein was determined by the Pierce and Suelter method (1977) and found to be 13.6 for a 1% solution.

Kinetics. Measurement of the substrate specificity factor (τ), or the partitioning between carboxylation and oxygenation, was performed using the simultaneous dual-labeling method of Jordan and Ogren (1981b) modified by Spreitzer et al., (1982), as recently described (Read & Tabita, 1992). Calculations were made using an O_2 concentration of 1.23 mM for 100% O_2-flushed reactions (Spreitzer et al., 1982). CO_2 concentrations were calculated from the concentration of HCO_3^-, using 6.12 as the pK' of CO_2/HCO_3^- at equilibrium.

Enzymes were preincubated in 10 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, and 50 mM Bicine, pH 8.0, for 1 h at 4 °C prior to determining the V_{CO_2}, K_{CO_2}, and K_{O_2}. Assays, conducted under both N_2 and 100% O_2, were initiated with the addition of activated enzyme (10 μg) in a reaction mixture containing 0.4 mM RuBP, 10 mM $MgCl_2$, 50 mM Bicine, pH 8.0, and various concentrations of $NaH^{14}CO_3$. Reactions were terminated after 30 s with propionic acid. Samples were dried at 60 °C and resuspended in 0.5 N HCl, and the ^{14}C dpm from 3-[^{14}C]phosphoglyceric acid produced was counted. V_{CO_2} and K_{CO_2} were calculated from the 100% N_2 reactions using Scatchard plots. K_{O_2} was derived from the ratio (R) of carboxylase activities under 100% N_2 versus 100% O_2 using the relationship of kinetic constants whereby $1/(R-1) = K_{O_2}/[O_2] + K_{O_2}[CO_2]/K_{O_2}[O_2]$ (Laing et al., 1975). The K_{O_2} was calculated from the intercept of this plot. V_{O_2} was derived from the specificity factor (τ), V_{CO_2}, K_{CO_2}, and K_{O_2}, using the equation $\tau = V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$.

The Michaelis constant for RuBP was determined by activating the purified enzymes at 25 °C for 20 min in 10 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$, and 50 mM Bicine, pH 8.0, under 100% N_2. To initiate the reaction, various amounts of RuBP were added to bring the final concentration from 8.25 to 105 μM. Reactions were terminated after 1 min with the addition of 50 mM $ZnSO_4$ in 0.05 N HCl. Following the addition of 1 M formic acid and 1 N HCl, samples were dried, resuspended, and counted as before. Estimates of the K_m for RuBP were computed from Scatchard plots.

Other Methods. The effect of 6-phosphogluconate on the carboxylase activity of the two hybrid enzymes was determined using a standard carboxylase activity assay (Whitman & Tabita, 1976). Enzymes were fully activated with 20 mM $NaHCO_3$ and 10 mM $MgCl_2$ and then incubated with various concentrations of 6-phosphogluconate (0–1.2 mM); carboxylase activity was then initiated by the addition of RuBP.

Carboxylase activities of the enzymes were also measured at various reaction times following preincubation with 0.8 mM RuBP or 10 mM $MgCl_2$ and 20 mM $NaHCO_3$. Preincubation was performed for 30 min at 30 °C prior to the addition of components required to complete the reaction mixture, which contained 10 mM $MgCl_2$, 20 mM $NaHCO_3$, and 0.8 mM RuBP.

Results

Expression of Active Hybrid Enzymes in E. coli. Hybrid enzymes containing *Synechococcus* large subunits and either *Cylindrotheca* N1 small subunits or *Olisthodiscus* small subunits were encoded by plasmids pVTAC223 and pANOLI, respectively. The recombinant chimeric proteins were synthesized in *E. coli*. Carboxylase activity was measured in the crude extracts. The specific activities of both hybrid enzymes (the pVTAC223 and pANOLI proteins) were 15% of the *Synechococcus* enzyme. The hybrid enzymes were purified to near-homogeneity in order to obtain more detailed information regarding their physical and biochemical characteristics. Previously established procedures for purifying the recombinant *Synechococcus* RubisCO (Tabita & Small, 1985; Lee & Tabita, 1990) proved effective for the isolation of the hybrid enzymes. The *Synechococcus* and the *Cylindrotheca* sp. N1 enzymes were also isolated, and all preparations were compared using nondenaturing and SDS–PAGE (Figure 1).

SDS–PAGE demonstrated that the *Cylindrotheca* sp. N1 small subunit and the *Olisthodiscus luteus* small subunit were each able to assemble in vivo with the *Synechococcus* L_8 octamer to form hybrid hexadecameric L_8S_8 enzymes. The large subunits of the chimeric enzymes (Figure 1A, lanes 2 and 3) migrate to the same position as that of the *Synechococcus* large subunit (Figure 1A, lane 1), exhibiting a molecular weight of 55 000. The small subunits, however, originating from *Cyl-*

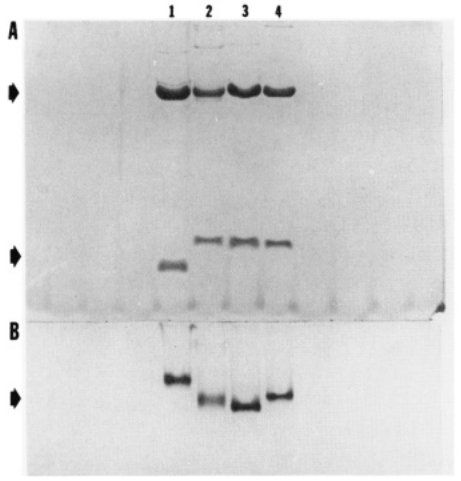

FIGURE 1: Polyacrylamide slab gel electrophoresis of purified heterologous RubisCO enzymes in the presence (A) and absence (B) of sodium dodecyl sulfate (0.1%). In both (A) and (B): lane 1, *Synechococcus* RubisCO; lane 2, pANOLI hybrid RubisCO; lane 3, the pVTAC223 hybrid RubisCO; lane 4, *Cylindrotheca* RubisCO. In (A), the arrows point to the positions of the large and small subunits, respectively. In (B), the arrow points to the position of the L_8S_8 native RubisCO.

indrotheca and *Olistodiscus*, migrate considerably slower than the small subunit of the *Synechococcus* enzyme (Figure 1A, lanes 2 and 3), reflecting their higher molecular weights (Boczar et al., 1990; Hwang & Tabita, 1991). The heterologous small subunits show identical electrophoretic mobility to the small subunits of the *Cylindrotheca* sp. N1 RubisCO (Figure 1A, lane 4). Densitometric analysis of Coomassie blue stained SDS–polyacrylamide gels containing large and small subunits of the pVTAC223 and pANOLI hybrid proteins yielded identical relative intensities when compared to densitometric scans of the large and small subunits of the *Synechococcus* holoenzyme, indicative of a molar ratio of large to small subunits of about 1:1, as previously determined for the *Synechococcus* enzyme (Andrews & Abel, 1981; Tabita & Small, 1985).

Under conditions of nondenaturing gel electrophoresis (Figure 1B), the proteins behaved somewhat aberrantly. The *Cylindrotheca* holoenzyme and the two hybrid enzymes, whose deduced molecular weight is greater than that of the *Synechococcus* L_8S_8, all migrate significantly faster than the cyanobacterial RubisCO. There is little difference in the net overall charge of the proteins; hence, the variance in migration must be due to subtle differences in the conformational state of the holoenzymes.

Determination of the CO_2/O_2 Specificity Factor and Kinetic Analyses of the Hybrid Enzymes. The CO_2/O_2 specificity factor for the *Synechococcus* enzyme, the *Cylindrotheca* enzyme, and the two hybrid enzymes was determined (Table I) using the established simultaneous dual-labeling assay (Jordan & Ogren, 1981b; Spreitzer et al., 1982). The specificity factor, τ, for the *Synechococcus* RubisCO was found to be 41, approximating the value previously reported for another cyanobacterial RubisCO (Jordan & Ogren, 1981a) and the value obtained for the *Synechococcus* 6301 enzyme by previous determinations in this laboratory (Lee et al., 1991; Read & Tabita, 1992). While the substrate specificity factor of the pANOLI hybrid enzyme did not differ significantly from the *Synechococcus* enzyme, the specificity factor of the pVTAC223 hybrid was 65, a value which is nearly 60% greater than the *Synechococcus* RubisCO.

The substrate specificity factor of the enzyme from *Cylindrotheca* sp. N1 was measured and found be 107. Likewise, we recently determined that RubisCOs from other nongreen algae possess unusually high specificity factors.[2] As a control, we determined the specificity factor of spinach RubisCO, and the value obtained, 77, was consistent with previously reported values (Jordan & Ogren, 1981a).

Table I: Kinetic Properties of Purified Cyanobacterial, Diatom, and Hybrid RubisCO Enzymes[a]

kinetic constant	source of enzyme			
	pBGL710[b]	Cyl.N1[c]	pVTAC223	pANOLI
τ	41.0 ± 4.4	107.1 ± 9.6	64.9 ± 4.6	37.4 ± 0.7
V_{CO_2}[d] (μmol min^{-1} mg^{-1})	2.23 ± 0.25	0.83 ± 0.07	0.11 ± 0.02	0.04 ± 0.01
V_{O_2}[e] (μmol min^{-1} mg^{-1})	0.25	0.15	0.04	0.01
V_{CO_2}/V_{O_2}	8.9	5.5	2.75	4.0
k_{cat}[f] (s^{-1})	2.57	0.97	0.13	0.05
K_{RuBP} (μM)	21.6 ± 3.8	8.7 ± 0.9	55.9 ± 2.7	60.0 ± 2.9
K_{CO_2} (μM)	142 ± 1	33 ± 5	85 ± 4	179 ± 6
K_{O_2} (μM)	664 ± 82	631 ± 43	1878 ± 207	1437 ± 173
K_{O_2}/K_{CO_2}	4.7	19.1	22.1	8.0

[a] Enzymes were purified and specificity factors (τ) and component kinetic parameters were determined as described under Experimental Procedures. [b] Recombinant *Synechococcus* enzyme. [c] *Cylindrotheca* sp. strain N1 enzyme. [d] V_{CO_2} values and standard errors were estimated by Scatchard plots. Lines were fitted by least-squares analysis. For each parameter, values represent the mean of at least three separate enzyme assays with sample (n − 1) standard deviations. [e] V_{O_2} values were derived according to the relationship $\tau = V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$ where K_{O_2} and K_{CO_2} represent mean K_m values. [f] k_{cat} for carboxylase reaction; calculated from V_{CO_2}.

The pVTAC223 hybrid enzyme exhibited maximal rates of carboxylation and oxygenation that were dramatically reduced compared to the *Synechococcus* enzyme. The V_{CO_2} and V_{O_2} values measured for the *Synechococcus* enzyme and the V_{CO_2}/V_{O_2} ratio are consistent with published values (Badger, 1980; Andrews & Abel, 1981; Voordouw et al., 1987; Read & Tabita, 1992). The V_{CO_2} for the hybrid pVTAC223 enzyme, however, was 0.11 μmol min^{-1} mg^{-1} with a k_{cat} of 0.13 s^{-1}, approximately 5% that of the wild-type *Synechococcus* RubisCO. The V_{O_2} for the pVTAC223 hybrid enzyme was 0.04 μmol min^{-1} mg^{-1}, or 16% that of the *Synechococcus* RubisCO.

The pANOLI hybrid enzyme also showed severely impaired maximal reaction rates for carboxylation and oxygenation. A V_{CO_2} of 0.04 μmol min^{-1} mg^{-1}, a k_{cat} of 0.05 s^{-1}, and a V_{O_2} of 0.01 μmol min^{-1} mg^{-1} were obtained for the pANOLI enzyme, representing 1% and 4% of the activity of the *Synechococcus* enzyme for carboxylation and oxygenation, respectively. The *Cylindrotheca* sp. N1 RubisCO exhibited maximal catalytic rates for carboxylation (0.83 μmol min^{-1} mg^{-1}, k_{cat} of 0.97 s^{-1}) and oxygenation (0.15 μmol min^{-1} mg^{-1}) that resemble what is normally observed for the higher plant enzyme (Bird et al., 1982; Jordan & Ogren, 1981a, 1984).

Comparisons of the Michaelis constants for the gaseous substrates and RuBP are also shown in Table I. The K_{O_2} of the pVTAC223 hybrid was nearly 3-fold higher (1878 μM) than the K_{O_2} of the *Synechococcus* enzyme (664 μM), while the K_{O_2} of the pANOLI hybrid was more than 2-fold higher (1437 μM). At the same time, the pVTAC223 enzyme exhibited a 40% reduction in its K_{CO_2} (85 μM) as compared to the *Synechococcus* enzyme (K_{CO_2} = 142 μM), whereas the pANOLI hybrid enzyme exhibited RubisCO (664 and 142 μM, respectively) are in basic agreement with previously reported values (Read & Tabita, 1992; Voordouw et al., 1987).

The K_{CO_2} of the *Cylindrotheca* enzyme (K_{CO_2} of 33 μM) correlated well with what is normally observed with the higher

[2] A preliminary report relative to the specificity factor of chromophytic and rhodophytic RubisCO and properties of hybrid enzymes recently appeared (Read & Tabita, 1992b).

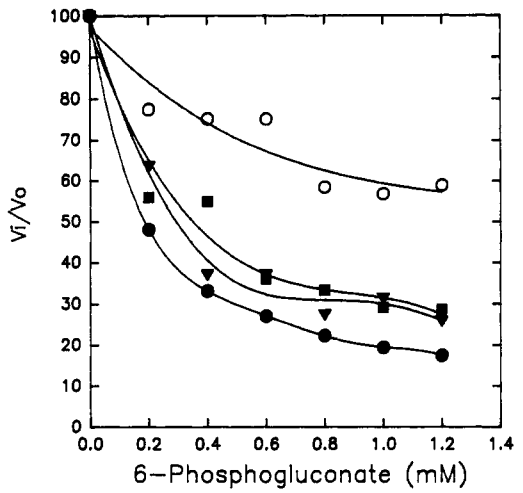

FIGURE 2: Sensitivity of activated enzymes to 6-phosphogluconate. *Synechococcus* RubisCO (○), *Cylindrotheca* RubisCO (●), the pVTAC223 hybrid RubisCO (▼), and the pANOLI hybrid RubisCO (■) were each activated with 10 mM Mg^{2+} and 20 mM HCO_3^- for 10 min at 30 °C prior to the addition of the indicated concentrations of 6-phosphogluconate. After incubation for an additional 5 min, the reaction was initiated with RuBP. Carboxylase activity was then measured for 5 min. V_0 and V_i are the activities obtained in the absence and presence of 6-phosphogluconate, respectively.

plant RubisCO (11–34 μM) (Jordan & Ogren, 1981a, 1983, 1984) but was slightly lower than values obtained for two other marine algal enzymes (Newman et al., 1989). In this latter study, RubisCOs from the marine algae *Olisthodiscus luteus* and *Griffithsia pacifica* were both found to possess K_{CO_2} values of 45 μM. In addition, the K_{O_2} of the *Cylindrotheca* enzyme (631 μM) was similar to values obtained for the higher plant enzyme (380–810 μM) (Jordan & Ogren, 1981a, 1983, 1984).

Michaelis constants for RuBP are exceedingly variable and show no general trends with respect to phylogeny or photosynthetic organization (Yeoh et al., 1981). It is worth noting, however, that the K_{RuBP} values of the two chimeric enzymes were both 2–3-fold greater than the K_{RuBP} of the *Synechococcus* enzyme; e.g., the K_{RuBP}'s were 56 and 60 μM for the pVTAC223 and pANOLI hybrids, respectively, as compared to 22 μm for the *Synechococcus* RubisCO. The K_{RuBP} for the *Cylindrotheca* RubisCO was considerably lower (K_{RuBP} of 9 μM).

Inhibition of the Hybrid Enzymes with 6-Phosphogluconate. The effect of the competitive inhibitor 6-phosphogluconate on the carboxylase activity of the *Synechococcus* wild-type enzyme and the two hybrid enzymes was examined (Figure 2). Before the carboxylase reaction was initiated with RuBP, enzymes were activated with 20 mM $NaHCO_3$ and 10 mM $MgCl_2$ and subsequently incubated with different concentrations of 6-phosphogluconate (0–1.2 mM). The *Cylindrotheca* N1 RubisCO and the two chimeric enzymes showed greater inhibition when titrated with increasing concentrations of 6-phosphogluconate than did the *Synechococcus* RubisCO. At 1.2 mM 6-phosphogluconate, the wild-type *Synechococcus* enzyme retained 60% of its activity, while the two hybrid enzymes retained approximately 30% of their original activities. At the same concentration, the *Cylindrotheca* N1 RubisCO retained less than 20% of its original activity.

Preincubation of the Hybrid Enzymes with RuBP. Incubation of many sources of RubisCO with RuBP prevents subsequent carbamylation of the enzyme by activator CO_2, stabilized by Mg^{2+}, resulting in a severe dimunition of the rate of catalysis. The *Synechecoccus* 6301 RubisCO, however, does not exhibit the RuBP-mediated inhibition typically observed with higher plant and bacterial two-subunit RubisCO enzymes

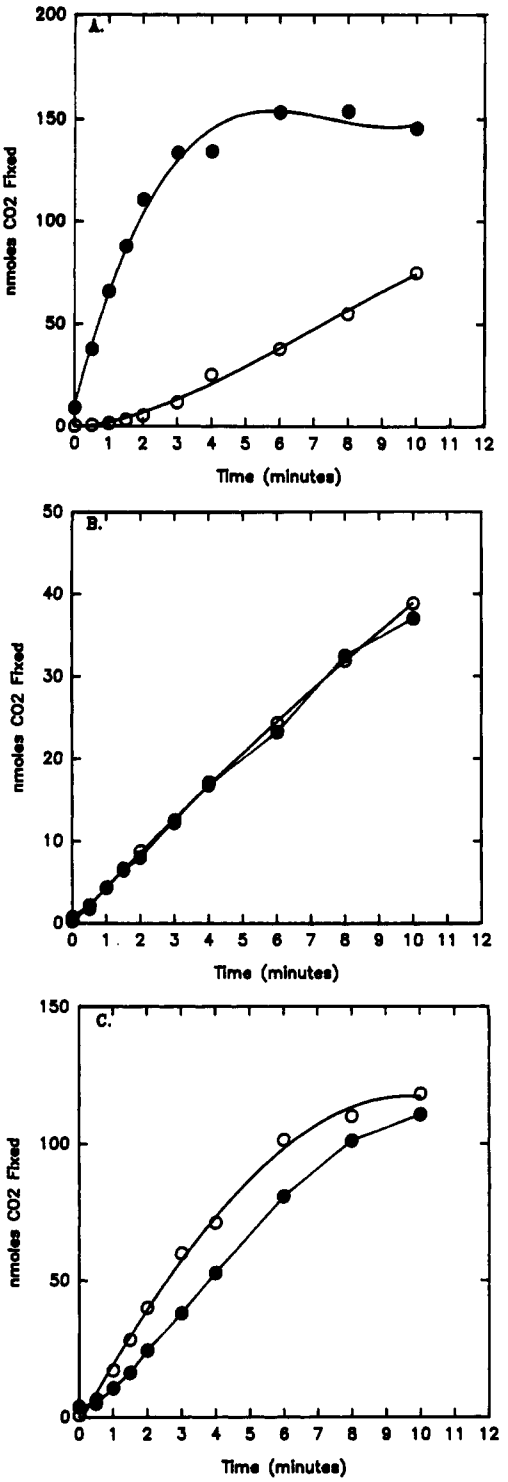

FIGURE 3: Preincubation of RubisCO with 0.8 mM RuBP (○) or 10 mM Mg^{2+}/20 mM HO_3^- (●). Carboxylase activity of (A) *Cylindrotheca* RubisCO (nanomoles of CO_2 fixed per minute), (B) the pVTAC223 hybrid RubisCO, and (C) the pANOLI hybrid RubisCO were measured as a function of reaction time after preincubation. Preincubation was performed for 30 min at 30 °C prior to the addition of components to complete the reaction mixture (10 mM Mg^{2+}, 20 mM HCO_3^-, and 0.8 mM RuBP).

(Lee et al., 1991). To study the effect of RuBP on the activation rate of the two hybrid enzymes, carboxylase activity was measured as a function of reaction time after preincubation either with 0.8 mM RuBP or with 20 mM HCO_3^- and 10 mM Mg^{2+}. While the *Cylindrotheca* RubisCO was markedly inhibited by RuBP preincubation (Figure 3A), the hybrid enzymes were either not inhibited (Figure 3B) or only slightly affected (Figure 3C).

Discussion

A number of hybrid RubisCO enzymes have been constructed in an attempt to define the possible role of the small subunit and to gain a better understanding of structure–function correlates of the carboxylase and oxygenase activities (Andrews & Lorimer, 1985; Andrews et al., 1984; Incharoensakdi et al., 1985; Lee et al., 1991; van der Vies et al., 1986) by combining RubisCO large and small subunits from different species. Using an in vitro constructed hybrid enzyme of *Synechococcus* large subunits and spinach small subunits, Andrews and Lorimer (1985) found that, while the partitioning between carboxylase and oxygenase functions remained unchanged, the carboxylase activity of the chimeric enzyme was reduced by 50% and the Michaelis constant for CO_2 was increased 2-fold as compared to the *Synechococcus* RubisCO. In the present investigation, heterologous small subunits derived from two eucaryotic marine organisms were combined with *Synechococcus* large subunits by in vivo expression and assembly of the enzyme in *E. coli*. Despite the high degree of homology (60% identity) in the amino acid sequence of the *Cylindrotheca* and *Olisthodiscus* small subunits, differential effects were conferred when the small subunits from the two algae were assembled with the L_8 octameric core from *Synechococcus*.

The catalytic competency of the chimeric enzymes was greatly reduced compared to the *Synechococcus* enzyme. In crude extracts, both heterologous constructs exhibited a specific activity that was roughly 15% that of the *Synechococcus* holoenzyme. When purified, the catalytic efficiency (denoted by the k_{cat}/K_c ratio) of the pVTAC223 hybrid was reduced by 1 order of magnitude, while the catalytic efficiency of the pANOLI hybrid was reduced by about 2 orders of magnitude. In addition, the k_{cat}/K_{RuBP} ratio for the pVTAC223 and pANOLI enzymes was reduced about 50- and 150-fold, respectively, when compared to the *Synechococcus* and *Cylindrotheca* enzymes. Perhaps, the decrease in the catalytic potential of the hybrid enzymes might be due to imprecise packing of the large and small subunits.

Of great significance, however, is that the substrate specificity factor of the pVTAC223 hybrid was enhanced nearly 60% over that of the *Synechococcus* enzyme, despite the deterioration in its catalytic potential. The specificity factor was not as high, however, as that of the *Cylindrotheca* enzyme. Nonetheless, these results provide evidence that this fundamental aspect of RubisCO catalysis may not be governed entirely by large subunits and further indicate that small subunits may be important in determining the partitioning between carboxylase and oxygenase functions, in agreement with recent speculations inferred from crystallographic analyses (Knight et al., 1990; Schneider et al., 1990). In addition, these results are in agreement with recent findings which indicate that factors other than large subunits may contribute or influence the substrate specificity factor (Chen et al., 1990). The major cause for the variation in the pVTAC223 enzyme, compared to the *Synechococcus* holoenzyme, appears to be due to a nearly 5-fold increase in the K_{O_2}/K_{CO_2} ratio, brought about by the nearly 3-fold increase in K_{O_2} and a 40% reduction in K_{CO_2}. While the K_{O_2}/K_{CO_2} ratio of the pVTAC223 hybrid enzyme resembles that of the *Cylindrotheca* enzyme, the K_{CO_2} and K_{O_2} values of the *Cylindrotheca* RubisCO are both about 66% lower than the K_{CO_2} and K_{O_2} values of the hybrid enzyme.

The pANOLI hybrid RubisCO exhibited a substantial increase in K_{O_2} relative to the *Synechococcus* enzyme (about 2-fold). This potential benefit was mitigated by the increase (nearly 30%) in its K_{CO_2}. Consequently, the slight increase in the K_{CO_2} was not sufficient to overcome the 50% reduction in the V_{CO_2}/V_{O_2} ratio; thus, the substrate specificity factor of the pANOLI hybrid enzyme was not significantly different from that for *Synechococcus* RubisCO. Unfortunately, the *Olisthodiscus* holoenzyme was not available to us, and we were therefore unable to make comparisions with it.

The specificity factor of the *Cylindrotheca* RubisCO was found to be 107, a value which is considerably higher than that measured for terrestrial plants (Jordan & Ogren, 1981a, 1983a). While the K_{CO_2}/K_{O_2} ratio of the *Cylindrotheca* RubisCO resembles the ratio observed for the higher plant enzyme, the V_{CO_2}/V_{O_2} ratio is somewhat higher than what is observed for the plant enzyme. Thus, the higher specificity factor of the *Cylindrotheca* enzyme seems to be primarily determined by a lower oxygenase turnover, as the V_{O_2} is considerably lower than what is exhibited by most higher plant enzymes (Christeller & Laing, 1979; Jordan & Ogren, 1984).

Cylindrotheca and other nongreen algae are unique in that both *rbcL* and *rbcS* are chloroplast-encoded (Boczar et al., 1989; Douglas et al., 1990; Hwang & Tabita, 1989; Valentin & Zetsche, 1989) and the deduced amino acid sequences of the large and small subunits from *Cylindrotheca* show significant homology to sequences from chemolithioautotrophic (Andersen & Caton, 1987; Meijer et al., 1991) and purple non-sulfur photosynthetic bacteria (Gibson et al., 1991; Hwang & Tabita, 1991) and are much less homologous to large and small subunit sequences from cyanobacteria, green algae, and higher plants. Residues that have previously been identified as active-site residues that are conserved across species (Knight et al., 1990) are conserved in the diatom large subunit as well (Hwang & Tabita, 1991). In addition, there are several residues within key regions of the protein that are found only in nongreen algal RubisCO large subunits (Douglas et al., 1990; Hwang & Tabita, 1991; Valentin & Zetsche, 1989). Whether these residues contribute to any particular function is open to conjecture at this time.

Analysis of the response to the two hybrid enzymes, in the presence of substrate RuBP, and evaluation of the catalytic capacity of the enzymes in the presence of the competitive inhibitor 6-phosphogluconate indicated that small subunits may influence very distinct aspects of the catalytic process. Both of the chimeric enzymes were unaffected by the presence of RuBP in preincubations, a response which reflects what is observed for the *Synechococcus* enzyme (Lee et al., 1991), the enzyme from another species of cyanobacteria (Andrews & Abel, 1981), the *R. rubrum* L_2 (Whitman et al., 1979), and the other single-subunit form II enzyme of *R. sphaeroides* (Gibson & Tabita, 1979). This is certainly unlike the dramatic RuBP-induced inhibition obtained for the enzyme from terrestrial plants (Jordan & Chollet, 1983), green algae (Jordan & Ogren, 1983), the bacterial L_8S_8 enzyme (Tabita & McFadden, 1974; Gibson & Tabita, 1977), and the diatom enzyme investigated here. These results are compatible with previous studies performed with hybrid bacterial enzymes (Lee et al., 1991) and demonstrate that the presence of small subunits from different organisms does not change the response to RuBP preincubation. The response of the unactivated enzymes to RuBP thus appears to be governed exclusively by the large subunits. Evidence from the presence investigation, along with the findings of Lee et al. (1991), does imply, however, that the binding of the competitive inhibitor 6-phosphogluconate is affected by small subunits. The increased sensitivity (nearly 2-fold) to 6-phosphogluconate conferred by the foreign small subunits derived from both *Cylindrotheca* and *Olisthodiscus* implies that there is a qualitative change

in the active site of the enzyme resulting from the heterologous assembly.

To summarize, the major finding of this study is the substantial increase in the substrate specificity factor of the *Synechococcus* catalytic large subunits conferred by *Cylindrotheca* small subunits. It is apparent that catalytic competency depends on subtle changes in active-site geometry which may be influenced by the binding of small subunits. Recent comparisons of the crystal structures of the L_2 and L_8S_8 forms of RubisCO indicate that the small subunits may interact with one of the α-helices in the C-terminal portion of the large subunit polypeptide (Knight et al., 1990). These investigators further suggest that such interactions may induce conformational changes in the active site to modulate substrate binding and, possibly, the substrate specificity factor, results which are compatible with the findings reported in this investigation. A direct determination of the tertiary structure of the pVTAC223 hybrid enzyme should thus yield important insights relative to the catalytic effects noted here. Obviously, it would be advantageous to increase the substrate specificity factor of RubisCO without having to sacrifice its catalytic potential. Perhaps, biological selection of second-site suppressor mutations, using a recently described system (Falcone & Tabita, 1991), will allow for the generation of mutant enzymes with both a high k_{cat} for carboxylation and an increased substrate specificity factor. One would also like to be able to identify specific residues that are responsible for inducing conformational and/or chemical alterations at the active site. That this is possible is suggested by previous findings whereby single-site mutations in the *Synechococcus* small subunits were also found to affect the catalytic potential and the Michaelis constants of the *Synechococcus* holoenzyme (Read & Tabita, 1992). The present study suggests that strategies for genetic manipulation of RubisCO may indeed become developed for increasing the CO_2/O_2 specificity factor (Chen & Spreitzer, 1989; Chen et al., 1988; Smith et al., 1990) but should not be confined to large subunits alone, as indicated by the results of this and other studies (Chen et al., 1990; Read & Tabita, 1992).

References

Andersen, K., & Caton, J. (1987) *J. Bacteriol. 169*, 4547–4558.

Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Branden, C., & Lorimer, G. H. (1989) *Nature 337*, 229–234.

Andrews, T. J., & Abel, K. M. (1981) *J. Biol. Chem. 256*, 8445–8451.

Andrews, T. J., & Ballment, B. (1983) *J. Biol. Chem. 258*, 7514–7518.

Andrews, T. J., & Lorimer, G. H. (1985) *J. Biol. Chem. 260*, 4632–4636.

Andrews, T. J., & Lorimer, G. H. (1987) *Biochem. Plants 10*, 131–218.

Andrews, T. J., Greenwood, D. M., & Yellowees, D. (1984) *Arch. Biochem. Biophys. 234*, 313–317.

Badger, M. R. (1980) *Arch. Biochem. Biophys. 201*, 247–254.

Bird, I. F., Cornelius, M. J., & Keys, A. J. (1982) *J. Exp. Bot. 33*, 1004–1013.

Boczar, B. A., Delaney, T. P., & Cattolico, R. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4996–4999.

Chen, Z., & Spreitzer, R. J. (1989) *J. Biol. Chem. 264*, 3052–3053.

Chen, Z., Chastain, C. J., Al-Abed, S. R., Chollet, R., & Spreitzer, R. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4696–4699.

Chen, Z., Green, D., Westhoff, C., & Spritzer, R. J. (1990) *Arch. Biochem. Biophys. 283*, 60–67.

Christeller, J. T., & Laing, W. A. (1979) *Biochem. J. 183*, 747–750.

Douglas, S. E., Durnford, D. G., & Morden, C. W. (1990) *J. Phycol. 26*, 500–508.

Estep, S. E., Tabita, F. R., & Van Baalen, C. (1978) *J. Phycol. 14*, 183–188.

Falcone, D. L., & Tabita, F. R. (1991) *J. Bacteriol. 173*, 2699–2108.

Gibson, J. L., & Tabita, F. R. (1977) *J. Biol. Chem. 252*, 943–949.

Gibson, J. L., & Tabita, F. R. (1979) *J. Bacteriol. 140*, 1023–1027.

Gibson, J. L., Falcone, D. L., & Tabita, F. R. (1991) *J. Biol. Chem. 266*, 14646–14653.

Gutteridge, S. (1991) *J. Biol. Chem. 266*, 7359–7362.

Horecker, B. L., Hurwitz, J., & Weissbach, A. (1958) *Biochem. Prep. 6*, 83–90.

Hudson, G. S., Mahon, J. D., Anderson, P. A., Gibbs, M. J., Badger, M. R., Andres, T. J., & Whitefield, P. R. (1990) *J. Biol. Chem. 265*, 808–814.

Hwang, S. R., & Tabita, F. R. (1989) *Plant Mol. Biol. 13*, 69–79.

Hwang, S. R., & Tabita, F. R. (1991) *J. Biol. Chem. 266*, 6271–6279.

Incharoensakdi, A., Takabe, T., Takabe, T., & Akazawa, T. (1985) *Biochem. Biophys. Res. Commun. 126*, 698–704.

Jordan, D. B., & Ogren, W. L. (1981a) *Nature 291*, 513–515.

Jordan, D. B., & Ogren, W. L. (1981b) *Plant Physiol. 67*, 237–245.

Jordan, D. B., & Chollet, R. (1983) *J. Biol. Chem. 258*, 13752–13758.

Jordan, D. B., & Ogren, W. L. (1983) *Arch. Biochem. Biophys. 227*, 425–433.

Jordan, D. B., & Ogren, W. L. (1984) *Planta 161*, 308–313.

Knight, S., Andersson, I., & Branden, C. I. (1990) *J. Mol. Biol. 215*, 113–160.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 254*, 367–382.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Laing, W. A., Ogren, W. L., & Hageman, R. H. (1975) *Biochemistry 14*, 2209–2275.

Lee, B., & Tabita, F. R. (1990) *Biochemistry 29*, 9352–9357.

Lee, B., Read, B. A., & Tabita, F. R. (1991) *Arch. Biochem. Biophys. 291*, 263–269.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, p 440, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mead, D. A., Szczesna-Skorupa, E., & Kemper, B. (1986) *Protein Eng. 1*, 67–74.

Meijer, W. J., Arnberg, A. C., Enequist, H. G., Terpstra, P., Lidstrom, M. E., & Dijkhuizen, L. (1991) *Mol. Gen. Genet. 225*, 320–330.

Newman, S. M., Derocher, J., & Cattolico, R. A. (1989) *Plant Physiol. 91*, 939–946.

Pierce, J., & Suelter, C. H. (1977) *Anal. Biochem. 82*, 478–480.

Read, B. A., & Tabita, F. R. (1992a) *Biochemistry 31*, 519–525.

Read, B. A., & Tabita, F. R. (1992b) *FASEB J. 6*, A209.

Smith, H. B., Larimer, F. W., & Hartman, F. C. (1990) *J. Biol. Chem. 265*, 1243–1245.

Smrcka, A. V., Ramage, R. T., Bohnert, H. J., & Jensen, R. G. (1991) *Arch. Biochem. Biophys. 286*, 6–13.

Spreitzer, R. J., Jordan, D. B., & Ogren, W. L., (1982) *FEBS Lett. 148*, 117-121.
Tabita, F. R. (1988) *Microbiol. Rev. 52*, 155-189.
Tabita, F. R., & McFadden, B. A. (1974) *J. Biol. Chem. 249*, 3459-3464.
Tabita, F. R., & Small, C. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6100-6103.
Valentin, K., & Zetsche, K. (1989) *Curr. Genet. 16*, 203-209.
van der Vies, S. M., Bradley, D., & Gatenby, A. A. (1986) *EMBO J. 5*, 2439-2444.
Voordouw, G., De Vries, P. A., Van den Berg, W. A. M., & De Clerk, E. P. J. (1987) *Eur. J. Biochem. 163*, 591-598.
Whitman, W., & Tabita, F. R. (1976) *Biochem. Biophys. Res. Commun. 71*, 1034-1039.
Yeoh, A., & Kitaoka, S. (1985) *Biochem. Biophys. Res. Commun. 131*, 1075-1079.

Analysis of the Two-State Behavior of the Thermal Unfolding of Serum Retinol Binding Protein Containing a Single Retinol Ligand†

Donald D. Muccio,*,‡ D. Vincent Waterhous,‡ Fred Fish,‡ and Christie G. Brouillette§
Department of Chemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294, and Department of Organic Chemistry and Biochemistry Research, Southern Research Institute, Birmingham, Alabama 35205
Received September 3, 1991; Revised Manuscript Received March 19, 1992

ABSTRACT: Through the use of CD and DSC, the thermal unfolding of holo serum retinol binding protein containing a single, tightly bound retinol ligand was studied at pH 7.4. The DSC endotherm of the holoprotein ([retinol]/[protein] = 1) was asymmetric about the transition temperature of 78 °C. Using changes in ellipticity at 230 nm, the thermal unfolding curve was also asymmetric about the inflection point centered near 78 °C. van't Hoff enthalpies were determined by three means and compared to the calorimetric enthalpy (ΔH_{cal}) of 200 kcal/mol. A van't Hoff enthalpy of 190 kcal/mol was determined from the dependence of transition temperature on the concentration of the ligand-bound protein. This value agreed well with the van't Hoff enthalpies found from fits of the DSC (ΔH_{vH} = 184 kcal/mol) and spectroscopic (ΔH_{vH} = 181 kcal/mol) curves to a two-state thermodynamic model that included ligand dissociation (NR $\rightleftharpoons$ U + R, where NR is the native holoprotein, U is the unfolded apoprotein, and R is retinol). Poor agreement was obtained with a two-state model that ignored ligand dissociation (N $\rightleftharpoons$ U). Furthermore, the NR $\rightleftharpoons$ U + R model accounted for the asymmetry in both CD and DSC transitions and yielded a much improved fit of the data over the N $\rightleftharpoons$ U model. From these considerations and simulations on other equilibrium models, it is suggested that the NR $\rightleftharpoons$ U + R model is the simplest model that describes the thermal unfolding of this ligand-bound protein. Using an averaged van't Hoff enthalpy determined from fits of DSC and CD data, the cooperativity of this process was 0.925, indicating that the unfolding of the holoprotein is nearly two-state.

Due to the low water solubility of vitamin A, the biological actions of this vitamin are often mediated through the interactions with proteins (Blomhoff et al., 1990). Human serum retinol binding protein (SRBP)[1] is the vitamin A transport protein that is normally complexed to transthyretin (TTR) when charged with the vitamin (Peterson & Rask, 1971; Goodman, 1984; Blaner, 1989). The hydrophobic binding pocket within SRBP protects vitamin A from oxidation and at the same time minimizes the cytotoxic effects of this fat-soluble vitamin. SRBP is homologous to several proteins that are small hydrophobic molecule transporters including β-lactoglobulin, apolipoprotein D, α_1-microglobulin, α_1-acid glycoprotein, bilin binding protein, BG protein from olfactory epithelium, and others (Godovac-Zimmermann, 1988). Together these proteins form a new protein superfamily.

X-ray crystallography (Newcomer et al., 1984; Cowan et al., 1990) has revealed that SRBP (21 kilodaltons) contains a single globular domain with a 40-Å diameter. The protein has eight antiparallel β-sheets and one small segment of α-helix. The tertiary structure of the protein forms a β-barrel that is flattened at one end. Retinol is tightly bound (K_d = 7.5×10^{-8} M) to the protein by noncovalent interactions (Noy & Xu, 1990a) and is sandwiched in the center of the β-barrel. The trimethylcyclohexenyl ring of retinol is buried deep within the flattened portion of the protein. The polyene chain of retinol extends throughout the hydrophobic core of SRBP and is surrounded by several aromatic amino acids. The hydroxyl end group of retinol is close to the cleft of the protein near the external aqueous environment.

Most small, single-domain proteins unfold in a highly cooperative manner that can be best represented by a two-state equilibrium process without intermediates (Lumry et al., 1966; Privalov, 1979; Kim & Baldwin, 1982, 1990). Unfolding studies on single-domain proteins with noncovalently bound

†This research was supported by the National Institutes of Health (P01 CA 28103-08) and the National Science Foundation (DIR-8820511). D.V.W. was supported by a T32 training grant from the National Institutes of Health (EY07033-12).
*Address correspondence to this author.
‡University of Alabama at Birmingham.
§Southern Research Institute.

[1] Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; SRBP, serum retinol binding protein; TTR, transthyretin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T_{max}, temperature of DSC maximum or inflection point in CD; T_m, temperature at which the equilibrium constant = 1; $T_{1/2}$, temperature at which the fraction of unfolded species = 0.5.

0006-2960/92/0431-5560$03.00/0